Isolation and biochemical diagnosis of cell lines of groundnut (*Arachis hypogaea* L) selected on glyphosate†

Mukesh Jain,[1] Raj K Bhatnagar[2] and Neera B Sarin[1]*

[1] *School of Life Sciences, Jawaharlal Nehru University, New Delhi 110 067, India*

[2] *International Centre for Genetic Engineering and Biotechnology, New Delhi 110 067, India*

Abstract: Three cell lines of groundnut (*Arachis hypogaea* L), an important oilseed legume, were selected on glyphosate using in-vitro culture techniques. The cell lines isolated through single as well as stepwise selection procedures showed *c* 20-fold increase in glyphosate tolerance as compared to the unselected control cell line. Studies on the biochemical mechanism of glyphosate tolerance in these cell lines showed a significant increase in the total extractable activity of the target enzyme, 5-*enol*pyruvyl shikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19), which was further confirmed with immunological data. The over-expressed EPSP synthase activity was, however, subject to inhibition by glyphosate *in vitro*. Two other key regulated enzymes of the shikimic acid pathway, 3-deoxy-D-arabino heptulosonate 7-phosphate (DAHP) synthase (EC 4.1.2.15) and chorismate mutase (CM) (EC 5.4.99.5) did not show any change in specific activity in the selected cell lines. The enhanced activity of EPSP synthase in the tolerant cell lines was found to be stably inherited in the absence of selection pressure.

Keywords: *Arachis hypogaea*; chorismate mutase; DAHP synthase; EPSP synthase; glyphosate; plant cell cultures

1 INTRODUCTION

Groundnut is an important oilseed legume crop of India, occupying the first position both with regard to area under cultivation and production. It occupies about 45% of the total cultivated land and yields about 55% of the oilseed produce. Groundnut is particularly sensitive to losses due to weed competition, primarily because of its slow growth, short stature, rainfed and pegging growth habit. These features also make mechanical weeding very difficult. In India, yield losses of groundnut due to weeds alone may be as high as 35–88%.[1]

Glyphosate [*N*-(phosphonomethyl)glycine] is a broad-spectrum, non-selective, foliar-applied, post-emergence, systemic herbicide with low mammalian toxicity and few residue problems. It is indicated for use in the control of a great variety of annual, biennial and perennial grasses, sedges and other weeds in various crops as well as non-crop areas. Glyphosate exhibits several desirable properties such as tight binding to soil, low soil mobility and rapid mineralization in the soil microenvironment, and, therefore, is at present the most extensively used herbicide worldwide. Glyphosate is a potent inhibitor of the shikimic acid pathway, a site of action unique amongst the herbicides. It acts by specifically inhibiting the target enzyme 5-*enol*pyruvyl shikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19), culminating in the arrest of protein synthesis and the prevention of secondary metabolism.[2] This inhibitor–enzyme complex has also received much academic attention because about 20% of the total carbon fixed as the photosynthate traverses the shikimic acid pathway.[3] While tolerance to glyphosate could (presumably) be acquired at the cellular level by its reduced uptake, degradation or conjugation, identification of EPSP synthase as the molecular target has allowed firm conclusions to be drawn with reference to the mode of action of this herbicide. Several micro-organisms and plant cell lines selected for glyphosate tolerance have been reported. In cell lines of carrot, tobacco, *Petunia, Corydalis sempervirens, Catharanthus roseus*, tomato, chicory and in a plastid-free mutant cell line of the phytoflagellate *Euglena gracilis*, glyphosate tolerance has been attributed to

* Correspondence to: Neera B Sarin, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110 067, India
E-mail: neera@jnuniv.ernet.in
† Based on a paper presented at the XVI Asian-Pacific Weed Science Society Conference, Kuala Lumpur, Malaysia, 8–12 September 1997
Contract/grant sponsor: University Grants Commission, New Delhi, India; contract/grant number: 3-36/94
Contract/grant sponsor: Council of Scientific and Industrial Research, New Delhi, India
(*Received 25 October 1998; accepted 22 April 1999*)

over-expression of the glyphosate-sensitive form of EPSP synthase.[4–12] In contrast, a herbicide-insensitive form of the enzyme has been reported in the bacteria *Salmonella typhemurium, Aerobacter aerogenes, Klebsiella pneumoniae, Agrobacterium tumefaciens, Euglena gracilis*, the bluegreen alga *Anabaena variabilis* and in a maize cell culture line.[13–19]

In the present investigation, we have isolated glyphosate-tolerant cell lines of groundnut (*Arachis hypogaea* L) by using in-vitro culture techniques and carried out investigations to understand the biochemical and molecular mechanism of glyphosate tolerance. The utility of selected cell culture lines towards synthesis of a practical approach for obtaining glyphosate-resistant (transgenic) plant lines has also been discussed.

2 MATERIALS AND METHODS

2.1 Chemicals and biochemicals

All the chemicals used were obtained from Sigma Chemical Co, St. Louis, MO and were of analytical grade. The barium salt of shikimate-3-phosphate was prepared according to Coggins *et al.*[20] Chorismic acid was prepared following the method of Gibson.[21]

2.2 Plant material and in-vitro cultures

Shelled groundnut seeds (*A hypogaea* var JL 24) were washed thoroughly under running tap water, surface-sterilized for 8–10 min in mercury (II) chloride (1 g litre^{-1}) and rinsed at least thrice in sterile water. Seeds were germinated aseptically on filter paper bridges soaked in liquid MS (Murashige and Skoog) medium fortified with sucrose (25 g litre^{-1}) and *myo*inositol, (100 mg litre^{-1}; pH 5.8).[22] The plantlets (12–14 days old) were used for issuing explants for initiation of in-vitro cultures. Leaf callus cultures were obtained on semi-solid MS medium supplemented with 1 mg litre^{-1} each of 6-benzylaminopurine and 1-naphthalene acetic acid. The suspension cultures were obtained by growing friable green callus on liquid MS medium (composition as above) with continuous shaking on a gyratory shaker at 100–120 strokes min^{-1}. All the in-vitro cultures were maintained at 25 (±2) °C, under cool white fluorescent light (1200 μW cm^{-2}) with a 16-h photoperiod and subcultured on the same medium every fortnight.

2.3 Selection of glyphosate-tolerant cell lines

The callus and suspension cultures of groundnut were screened for the 50% lethal dose (LD_{50}) of glyphosate. Three cell lines were selected: (i) GR1, selected by challenging the callus initially at a lethal dose of 1.5 mM glyphosate (single-step selection), (ii) GR2, selected at a sub-lethal dose of 0.3 mM glyphosate (multistep selection) and (iii) GRS1, selected from a suspension culture mutagenized at 10 Gy with γ radiation, emitted from a ^{60}Co source. The mutant/variant cells were selected for glyphosate tolerance at a lethal dose of 2.0 mM glyphosate. The mutagenized cells were plated on semi-solid medium containing 2.0 mM glyphosate. The surviving, actively growing, green microcalli were picked up after two weeks and put on herbicide-free medium for two weeks to allow the cells to recover, and finally transferred to the selection medium. Isopropylamine salt of glyphosate was used for all experiments. The glyphosate stock was prepared by titrating with sodium hydroxide (1 M) until it dissolved completely and making up the final volume with water. The solution was adjusted to neutral pH, filter-sterilized by passage twice through 0.45-μm Whatman filters and added to the autoclaved medium to the desired concentration.

The cell lines selected as above were maintained as described and subcultured after every 15 days. After about six months, giberellic acid (1 mg litre^{-1}) was also included in the medium. This helped maintain a healthy green colour of the callus under selection. The selection pressure was increased slowly over a period of two years.

2.4 Analytical methods

Phosphatase activity was assayed by measuring the enzymatic formation of *p*-nitrophenol from *p*-nitrophenylphosphate, as described by Malamy and Horecker.[23] The background, non-specific phosphatase activity was determined for correction of the total extractable EPSP synthase activity, since the procedure described measures the rate of appearance of P*i*, detected spectrophotometrically by the Malachite Green binding assay. EPSP synthase was assayed by the method of Forlani *et al*[19] with a slight modification, in that the extraction buffer and the reaction mixture contained 5.0 μM sodium *meta*vanadate instead of ammonium heptamolybdate for inhibition of the background phosphatase activity. DAHP synthase and chorismate mutase were assayed by the procedure of Sharma *et al.*[24] The method of Bradford was followed for protein quantification using bovine serum albumin as the standard.[25]

2.5 Immunodetection of EPSP synthase

An immunoassay was developed for *Arachis* EPSP synthase using the rabbit polyclonal antiserum developed against *Bacillus subtilis* EPSP synthase enzyme. The polyclonal antibodies were a kind gift from Dr Raj K Bhatnagar, ICGEB. The immuno dot blots were performed, essentially according to Harlow and Lane.[26] The primary and secondary (anti-IgG HRP conjugate) antibodies were used at dilutions of 1:10 000 and 1:7,500 respectively and 4-chloro-1-naphthol was used to develop the HRP label. Immunoreactive EPSP synthase in the groundnut cell lines was quantified (ELISA) using the primary and secondary antibodies at dilutions of 1:5 000 and 1:7,500 respectively. Tetramethylbenzidine was used for detecting the HRP label and the plates were read at 450 nm.

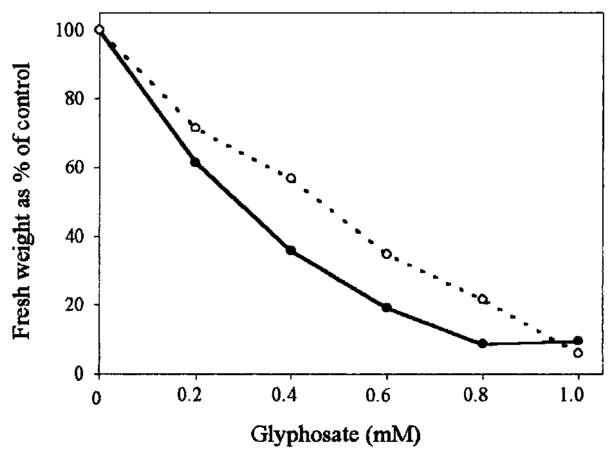

Figure 1. Effect of glyphosate on the growth of (—●—) germinating embryos and (···○···) callus cultures. Growth of seedlings and callus was determined as fresh weight increase after 15 days, the control values being 5.618 ($\pm$0.045) g FW per 10 seedlings and 2.852 ($\pm$0.015), calculated as g FW accumulated per g FW inoculated, respectively.

3 RESULTS AND DISCUSSION

3.1 Glyphosate-induced growth inhibition

Glyphosate is a foliar-applied herbicide but can also be absorbed by the roots when supplied in the nutrient solution in water cultures. The herbicidal effects of glyphosate on the growth of groundnut seedlings and in-vitro callus cultures were studied. The results show the quantitative effects of glyphosate in the form of an inhibition curve (Fig 1). Glyphosate at a concentration of 0.5 mM was enough to reduce the fresh weight gain of callus cultures by 50%. Embryo germination appeared to be more sensitive to glyphosate, since only about 0.3 mM herbicide was needed to arrest embryo germination and its subsequent development by 50%. Glyphosate, when present in sub-lethal concentration, resulted in systemic reduction in the size of groundnut seedlings, short and stumpy hypocotyls with a swollen base, reduced length of internodes, malformed leaves and a complete failure of the secondary root system. Under in-vitro conditions, the cells challenged with glyphosate showed a drastic decline in the growth rate, accompanied by a loss of colour initially, followed by a pronounced necrosis reaction.

The effect of external administration of aromatic amino acids in overcoming glyphosate toxicity was studied. The glyphosate-induced growth inhibition could be alleviated, though only partially, by exogenous administration of aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Tryp) (supplied either singly or in combination) (Figs 2a and b). The mixture of all the three aromatic amino acids could alleviate growth inhibition by 50–70% as compared to the controls. Effectiveness of single amino acids was in the order Phe > Tyr > Tryp, with the mixture Phe + Tyr being most effective. Successful reversal of glyphosate toxicity has earlier been shown for a wide variety of microorganisms and cultured plant cells, but only in the case of *Arabidopsis thaliana* (L)Heynh has this been shown for an intact, higher terrestrial plant.[9,27,28] Tricarboxylic acid cycle intermediates (particularly α-ketoglutarate, succinate and oxaloacetate) were also as effective as aromatic amino

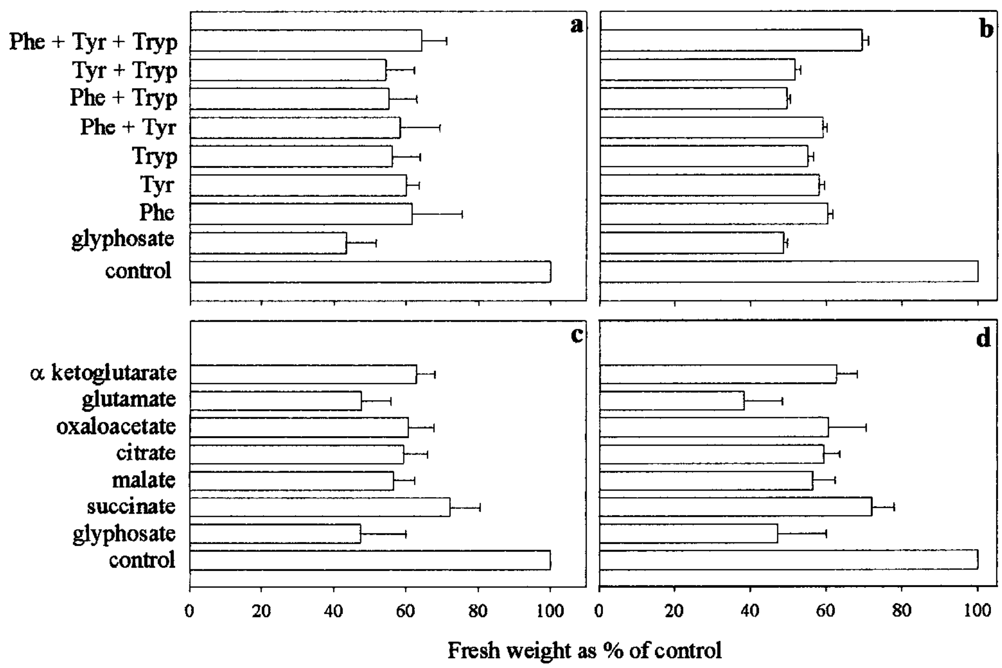

Figure 2. Alleviation of the inhibitory effects of glyphosate on (a and c) the growth of germinating embryos and (b and d) callus cultures by aromatic amino acids and tricarboxylic acid cycle intermediates. The control values were (a) 6.195 ($\pm$0.081) g FW per 10 seedlings, (b) 1.291 ($\pm$0.093) g FW accumulated per g FW inoculated, (c) 5.803 ($\pm$0.100) g FW per 10 seedlings and (d) 1.507 ($\pm$0.082) g FW accumulated per g FW inoculated. The aromatic amino acids and tricarboxylic acid cycle intermediates were supplemented at a final concentration of 1.0 and 2.0 mM respectively and glyphosate was added to a final concentration of 0.3 mM (a and c) and 0.5 mM (b and d). Horizontal bars are $\pm$SE.

acids in reversing glyphosate-induced growth inhibition (Figs 2c and d). Similar results have been reported in the case of suspension-cultured cells of *Daucus carota*.[29]

Inhibition of EPSP synthase in its mid-pathway position has been postulated to have two effects that seem to apply universally. The starvation of aromatic pathway end-products is compensated by the elevation of repressible enzymes and relief of allosteric enzymes from feedback inhibition occurring due to regulatory adjustments. This produces the second effect, an unrestrained entry of phospho*enol*pyruvate (PEP) and erythrose-4-phosphate (E4P) into the shikimic acid pathway. The metabolic manifestation of wasteful loss of PEP and ATP (*energy drain*) varies somewhat for every organism and the particular details of reversal (partial to complete) of glyphosate inhibition by aromatic amino acids are variable, reflecting the diversity of aromatic amino acid biosynthesis in nature.[30] The inhibition of plant cells in culture by glyphosate is characteristically reversed only partially by aromatic amino acids, as has also been shown in the earlier studies. It has been hypothesized that energy drain within the chloroplast compartment plays a significant role in the herbicidal events leading to the systemic death of higher plants.[31] Addition of exogenous tricarboxylic acid cycle intermediates increases the availability of PEP and E4P for aromatic biosynthesis, thereby overwhelming competitive inhibition at the enzyme target of glyphosate.

3.2 Selection of glyphosate-tolerant cell lines

Isolation of glyphosate-tolerant cell lines of groundnut has been described in the previous section. At present, GR1 and GR2 are growing at a concentration of 8.0 mM and GRS1 can tolerate up to 7.0 mM glyphosate. These cell lines grow at rates comparable to the control unselected cell line, as indicated by the fresh-weight gain of these cultures. The growth curves for the herbicide-sensitive as well as tolerant cell lines were typically sigmoid, with the midlog phase being reached 12–14 days after culture (data not shown). A comparison of the growth behaviour of the herbicide-tolerant cell lines in contrast to the sensitive, unselected cells challenged with increasing concentrations of glyphosate is shown in Fig 3. While growth of sensitive callus was inhibited by 50% at 0.5 mM glyphosate, about 10 mM glyphosate was required to achieve the same effect in the herbicide-tolerant cell lines. The herbicide-tolerant cell lines, therefore, showed *c* 20-fold increase in tolerance to glyphosate as compared to the unselected cell line.

3.3 Over-expression of EPSP synthase in glyphosate-tolerant cell lines

Glyphosate elicits herbicidal activity by inhibiting aromatic biosyntheses, the molecular target being the penultimate enzyme of the pre-chorismate common biosynthetic pathway, EPSP synthase. The glyphosate-selected cell lines continuously cultured in presence of the herbicide were found to have a specific activity of EPSP synthase higher than the unselected cell line (Fig 4a). Rabbit antiserum prepared against the *Bacillus subtilis* EPSP synthase was used to establish an assay for the immunoreactive protein in the groundnut cell lines. By visual comparison of the intensities of the peroxidase stain in a series of the crude protein extracts from the control and the glyphosate-selected cell lines, it could be perceived that the increased EPSP synthase specific activity corresponded closely with the enhanced amount of cross-reactive protein (data not shown). The relative abundance of immunoreactive EPSP synthase in

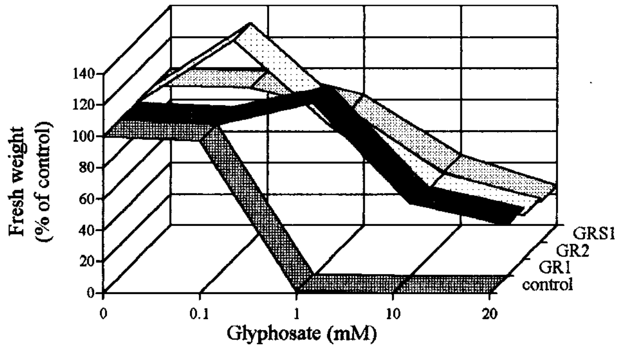

Figure 3. Effect of glyphosate on the fresh weight accumulation in herbicide-sensitive *vs* tolerant cell lines. The control values for sensitive, GR1, GR2 and GRS1 cells were 1.08 (±0.09), 1.30 (±0.11), 1.50 (±0.09) and 1.60 (±0.10) respectively, calculated as g FW accumulated per g FW inoculated.

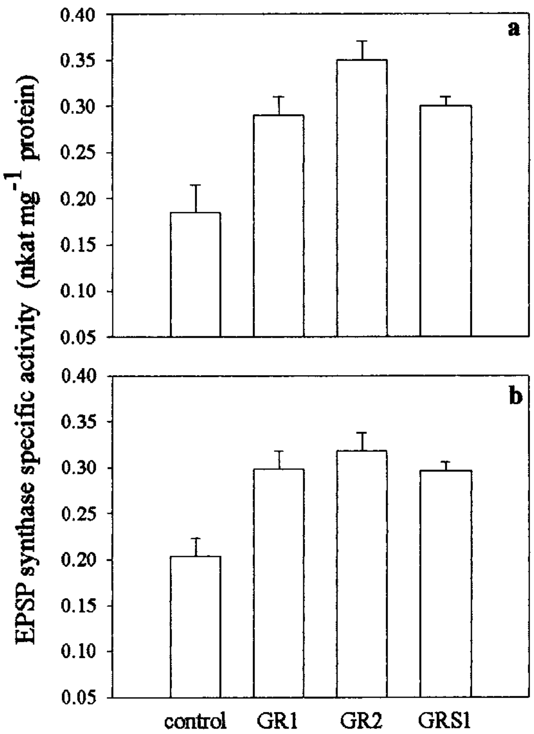

Figure 4. Over-expression of EPSP synthase in (a) glyphosate-sensitive *vs* tolerant cell lines selected on glyphosate-containing medium and (b) the same maintained on herbicide-free medium for two years. Vertical bars are ±SE.

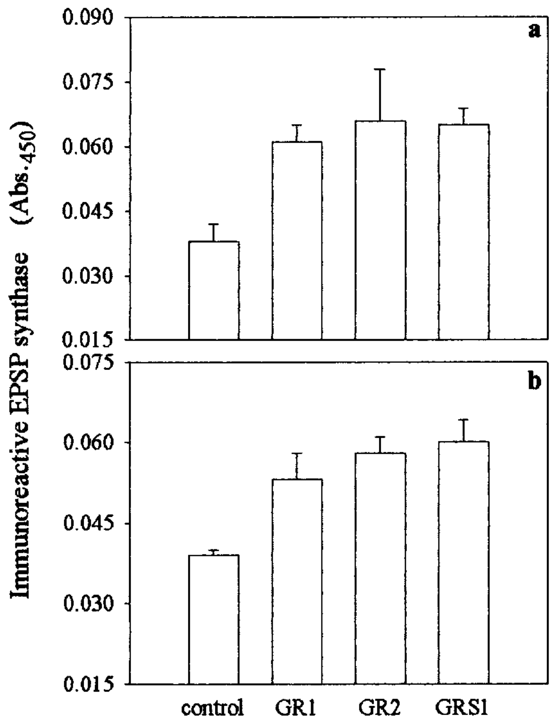

Figure 5. Over-expression of immunoreactive EPSP synthase in (a) glyphosate-sensitive *vs* tolerant cell lines selected on glyphosate-containing medium and (b) the same maintained on herbicide-free medium for two years. The primary and secondary (anti-IgG HRP conjugate) antibodies were used at dilutions of 1:5000 and 1:7,500 respectively. The HRP label was developed using tetramethyl benzidine. Vertical bars are ±SE.

glyphosate-sensitive and tolerant cell lines was quantified by ELISA, and the data presented indicate 60–75% increase in the antigen level in the herbicide-tolerant cell lines, comparing well with the 52–68% increase in the specific activity of the enzyme (Fig 5a).

Biochemical and immunological data strongly suggested selective overproduction of EPSP synthase in the glyphosate-selected cell lines of groundnut. The in-vitro enzyme activities from both glyphosate-sensitive and tolerant cell lines, were equally subject to inhibition by the herbicide glyphosate. Under standard in-vitro assay conditions of substrate saturation, EPSP synthase enzyme activity in the crude protein extracts was reduced by about 50% at 2 μM glyphosate in all the cell lines (Fig 6). However, the enzyme from both the sources needs to be purified for an accurate comparison of its physical, chemical and kinetic properties. The lack of differential effect of glyphosate on the in-vitro enzyme activity from glyphosate-sensitive and tolerant cell lines suggested that the EPSP synthase protein from both the sources was indeed similar. The herbicide tolerance thus achieved during the selection procedure could be due to gene amplification and/or enhanced expression of the original EPSP synthase gene.

The activities of two key regulated enzymes of the shikimic acid pathway, other than EPSP synthase, were also examined in glyphosate-tolerant cell lines.

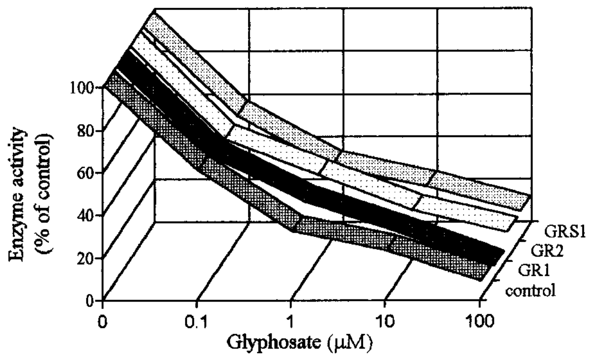

Figure 6. In-vitro inhibition of EPSP synthase activity by glyphosate. The control values for sensitive, GR1, GR2 and GRS1 cells were 0.22 (±0.01), 0.32 (±0.04), 0.36 (±0.00) and 0.30 (±0.02) nkat mg^{-1} protein respectively.

DAHP synthase catalyzes the first and the committed reaction of the aromatic biosynthesis involving condensation of PEP and E4P, thus sharing the substrate pool of EPSP synthase for its PEP requirement. The enzyme chorismate mutase converts chorismate to prephenic acid and terminates the common pre-chorismate trunk of the pathway. The specific activities of DAHP synthase and chorismate mutase were compared in glyphosate-sensitive and tolerant cell lines (Fig 7). No differences of significant magnitude were evident in the two types of cell line. It also seems difficult to attach any special significance to the somewhat elevated DAHP synthase activity in the GR1 cell line.

Pinto *et al*[32] reported an increase in the activity of

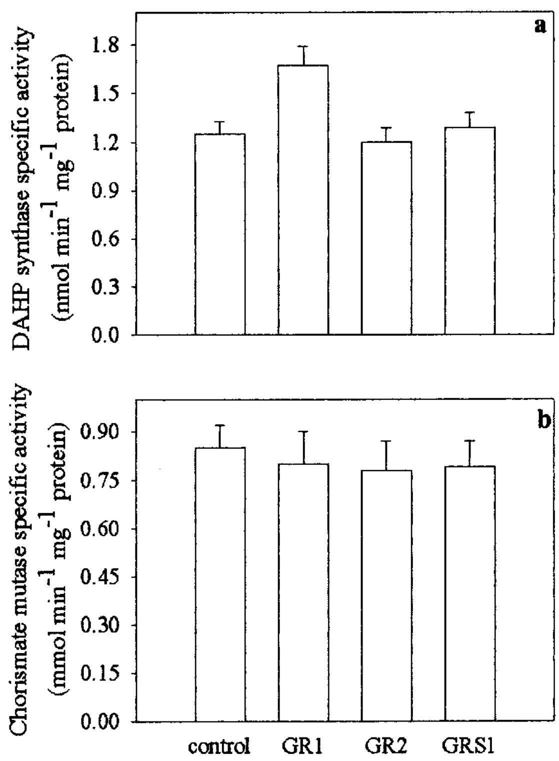

Figure 7. Specific activities of (a) DAHP synthase and (b) chorismate mutase in glyphosate-sensitive *vs* tolerant cell lines. Vertical bars are ± SE.

DAHP synthase, when potato (*Solanum tuberosum* L) cells grown in suspension cultures were treated with sublethal doses of glyphosate. The relative increase in the activity was a reflection of glyphosate concentration. Apparently, blocking the penultimate step in the pathway triggers a signal that causes the modulation of the activity of the first enzyme. The signal may be a simple release of the feedback inhibition operative at the first step, or a metabolite like L-arogenate.[33] Cole *et al*[34] observed that the root tips of wheat (*Triticum aestivum* L) yielded elevated specific activity of chorismate mutase in response to glyphosate presentation. Since the regulation of the shikimic acid pathway in higher plants is exerted by direct feedback inhibition rather than the modulation of the enzyme levels,[35] such effects are unlikely to be true derepression responses.

3.4 Stability of the selected phenotype

The cell lines were maintained on glyphosate-free medium for more than two years (nearly 60 culture cycles), before transferring them back onto the selection medium. All three cell lines were found to have retained the selected phenotype, and grew at rates comparable to the lines that were grown continuously on glyphosate-containing medium. Only marginal losses in the enhanced specific activity (Fig 4b) and in the content of immunoreactive EPSP synthase were detected (Fig 5b).

4 CONCLUSIONS

The past few decades have witnessed an alarming and devastating rise in the incidence of weed epidemics taking their toll on agricultural productivity. The available herbicides are not always sufficiently selective, thus contraindicating and limiting their therapeutic use during the active growth season of the crop. It has now become extremely difficult and costly to discover and develop new herbicides with favourable phytotoxic, selectivity and environment-friendly characteristics via random screening approaches. Recent advances in plant tissue culture and molecular biology techniques offer an advantage whereby the crop's tolerance towards an already existing safe herbicide can be achieved. This would save the expense associated with the discovery, development and marketing of a new product, passing on the reduced cost to the end user and offering a greater flexibility in the choice of herbicides and crops for the weed management programs. Only limited use has been made of classical breeding methods for developing herbicide-tolerant crops, due to the low occurrence of resistant individuals in natural populations and low selection pressures operative under field conditions. In-vitro culture systems allow screening of large cell populations in small volumes with short generation times. The uniformity of cell cultures also facilitates rapid selection under stringent selection conditions.

The present proposal was pursued so as to obtain a groundnut genotype for which glyphosate can be effectively and conveniently used as a part of weed-control practices. The glyphosate-selected cell lines were screened for the degree of herbicide tolerance as well as for the inheritance of the selected phenotype in absence of the selection pressure. The biochemical studies of the cell lines of groundnut selected on glyphosate confirm that EPSP synthase is the only enzyme target for glyphosate action and further that the aromatic biosynthesis pathway enzymes are not subject to coordinate control. Acquisition of glyphosate tolerance in the selected cell lines appears to be *via* overproduction of the target enzyme EPSP synthase. The lack of differential effect of glyphosate on the in-vitro enzyme activity from glyphosate-sensitive and tolerant cell lines suggested that the EPSP synthase proteins from both the sources are identical, and that the herbicide tolerance achieved during the selection procedure is (presumably) due to gene amplification and/or enhanced expression of the original EPSP synthase gene. Preliminary studies on the molecular mechanism of herbicide tolerance have revealed that the enhanced expression of EPSP synthase in the selected cell lines is associated with amplification of the EPSP synthase genes and higher transcriptional activity.

Work in our laboratory is currently directed towards cloning of the gene for glyphosate tolerance from the EPSP synthase-overproducing cell lines of groundnut. This gene would be of great interest in the study of spatial and temporal regulation patterns, the underlying mechanisms, and for developing glyphosate-resistant transgenic crop plants. The EPSP synthase gene (and a host of other genes known to confer herbicide resistance eg PAT, phosphinothricin acetyl transferase) are also being used as efficient selection markers for plant transformation studies. Having standardized a (direct) regeneration protocol for groundnut, from the cut hypocotyl surface, attempts are also under way to regenerate the herbicide-tolerant cell lines of groundnut.

ACKNOWLEDGEMENTS

The UGC grant No. 3-36/94 (SR II) to NBS, CSIR Senior Research Fellowship to MJ and the help of Project Fellow Ms Sunita Kaul is gratefully acknowledged.

REFERENCES

1 Reddy DVV, Narayana B and Rao AN, *Abstracts, Biological Conference, Indian Society of Weed Science*, Jabalpur, India, Abstract No 76 (1990).

2 Steinrucken HC and Amrhein N, The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. *Biochem Biophys Res Commun* **94**:1207–1212 (1980).

3 Haslam F, *The Shikimate Pathway* Halsted New York (1974).

4 Nafziger ED, Widholm JM, Steinrucken HC and Killmer JL, Selection and characterization of a carrot cell line tolerant to glyphosate. *Plant Physiol*, **76**:571–574 (1984).

5 Singer SR and McDaniel CN, Selection of glyphosate tolerance in tobacco calli and the expression of this tolerance in regenerated plants. *Plant Physiol* **78**:411–416 (1985).
6 Dyer WE, Weller SC, Bressan RA and Herrmann KM, Glyphosate tolerance in tobacco (*Nicotiana tabacum* L). *Plant Physiol* **88**:661–666 (1988).
7 Steinrucken HC, Schulz A, Amrhein N, Porter CA and Fraley RT, Overproduction of 5-enolpyruvylshikimate-3-phosphate synthase in a glyphosate-tolerant *Petunia hybrida* cell line. *Arch Biochem Biophys* **244**:169–178 (1986).
8 Smart CC, Johanning D, Muller G and Amrhein N, Selective overproduction of 5-enolpyruvylshikimate-3-phosphate synthase in a plant cell culture which tolerates high doses of the herbicide glyphosate. *J Biol Chem* **260**:16338–16346 (1985).
9 Cresswell RC, Fowler MW and Scragg AH, Glyphosate tolerance in *Catharanthus roseus*. *Plant Sci* **54**:55–63 (1988).
10 Smith CM, Pratt D and Thompson GA, Increased 5-enolpyruvykshikimic acid 3-phosphate synthase activity in a glyphosate-tolerant variant strain of tomato cells. *Plant Cell Rep* **5**:298–301 (1986).
11 Sellin C, Forlani G, Dubois J, Nielsen E and Vasseur J, Glyphosate tolerance in *Cichorium intybus* L var Magdebourg. *Plant Sci* **85**:223–231 (1992).
12 Reinboth S, Ortel B and Parthier B, Overproduction by gene amplification of the multifunctional *arom* protein confers glyphosate tolerance to a plastid-free mutant of *Euglena gracilis*. *Mol Gen Genet* **239**:416–424 (1993).
13 Stalker DM, Hiatt WR and Comai L, A single amino acid substitution in the enzyme 5-enolpyruvylshikimate-3-phosphate synthase confers resistance to the herbicide glyphosate. *J Biol Chem* **260**:4724–4728 (1985).
14 Schulz A, Sost D and Amrhein N, Insensitivity of 5-enolpyruvylshikimate phosphate synthase to glyphosate confers resistance to this herbicide in a strain of *Aerobacter aerogenes*. *Arch Microbiol* **137**:121–123 (1984).
15 Sost D and Amrhein N, Substitution of Gly-96 to Ala in the 5-enolpyruvylshikimate-3-phosphate synthase of *Klebsiella pneumoniae* results in a greatly reduced affinity for the herbicide glyphosate. *Arch Biochem Biophys* **282**:433–436 (1990).
16 Barry G, Kishore G, Padgette S, Taylor M, Kolacz K, Weldon M, Re D, Eichholtz D, Fincher K and Hallas L, Inhibitors of amino acid biosynthesis: Strategies for imparting glyphosate tolerance to crop plants, in *Biosynthesis and Molecular Regulation of Amino Acids in Plants*, ed by Singh BK, Flores HE and Shannon JC, American Society of Plant Physiologists. pp 139–145 (1992).
17 Reinbothe S, Nelles A and Parthier B, N-(Phosphonomethyl)-glycine (glyphosate) tolerance in *Euglena gracilis* acquired by either overproduced or resistant 5-enolpyruvylshikimate-3-phosphate synthase. *Eur J Biochem* **198**:365–373 (1991).
18 Powell HA, Kerby NW, Rowell P, Mousdale DM and Coggins JR, Purification and properties of a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase from the cyanobacterium *Anabaena variabilis*. *Planta* **188**:484–490 (1992).
19 Forlani G, Nielsen E and Racchi ML, A glyphosate-resistant 5-enolpyruvyl shikimate-3-phosphate synthase confers tolerance to a maize cell line. *Plant Sci* **85**:9–15 (1992).
20 Coggins JR, Boocock MR, Chauduri S, Lambert JML, Lumsden J, Nimmo GA and Smith DDS, The *arom* multifunctional enzyme from *Neurospora crassa*. *Methods Enzymol* **142**:325–338 (1987).
21 Gibson F, Preparation of chorismic acid. *Methods Enzymol* **17A**:362–364 (1970).
22 Murashige T and Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* **14**:473–497 (1962).
23 Malamy MH and Horecker BL, Purification and crystallization of the alkaline phosphatase of *Escherichia coli*. *Biochemistry* **3**:1893–1897 (1964).
24 Sharma R, Bhatnagar RK and Sarin NB, Differential expression of DAHP synthase and chorismate mutase isozymes during rhizogenesis of *Brassica juncea* cultures *in vitro*. *Physiol Plant* **88**:281–286 (1993).
25 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**:248–254 (1976).
26 Harlow E and Lane D, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, USA (1998).
27 Cole DJ, Mode of action of glyphosate –a literature review, in *The Herbicide Glyphosate*, ed by Grossbard E and Atkinson D, Butterworths, London. pp 48–74 (1985).
28 Gresshoff PM, Growth inhibition by glyphosate and reversal of its action by phenylalanine and tyrosine. *Australian J. Plant Physiol* **6**:177–185 (1979).
29 Killmer J, Widholm J and Slife F, Reversal of glyphosate inhibition of carrot cell culture growth by glycolytic intermediates and organic and amino acids. *Plant Physiol* **68**:1299–1302 (1981).
30 Fischer RS, Berry A, Gaines CG and Jensen RA, Comparative action of glyphosate as a trigger of energy drain in Eubacteria. *J Bacteriol* **168**:1147–1154 (1986).
31 Jensen RA, The shikimate/arogenate pathway: link between carbohydrate metabolism and secondary metabolism. *Physiol Plant* **66**:164–168 (1986).
32 Pinto JFBP, Dyer WF, Weller SC and Herrmann KM, Glyphosate induces 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase in potato (*Solanum tuberosum* L) cells growing in suspension culture. *Plant Physiol* **82**: 1040–1044 (1988).
33 Rubin JL and Jensen RA, Differentially regulated isozymes of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from seedlings of *Vigna radiata* [L] Wilezek. *Plant Physiol* **79**:711–719 (1985).
34 Cole DJ, Dodge AD and Caseley JC, Some biochemical effects of glyphosate on plant meristems. *J Exp Bot* **31**:1665–1974 (1980).
35 Gilchrist DG and Kosuge T, Aromatic amino acid biosynthesis and its regulation, in *The Biochemistry of Plants*, Vol 5, ed by Miflin BJ, Academic Press, New York, pp 507–531 (1980).